Bitter taste responses of gustducin-positive taste cells in mouse fungiform and circumvallate papillae

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Abstract

Bitter taste serves as an important signal for potentially poisonous compounds in foods to avoid their ingestion. Thousands of compounds are estimated to taste bitter and presumed to activate taste receptor cells expressing bitter taste receptors (Tas2rs) and coupled transduction components including gustducin, phospholipase Cβ2 (PLCβ2) and transient receptor potential channel M5 (TRPM5). Indeed, some gustducin-positive taste cells have been shown to respond to bitter compounds. However, there has been no systematic characterization of their response properties to multiple bitter compounds and the role of transduction molecules in these cells. In this study, we investigated bitter taste responses of gustducin-positive taste cells *in situ* in mouse fungiform (anterior tongue) and circumvallate (posterior tongue) papillae by using transgenic mice expressing green fluorescent protein in gustducin-positive cells. The overall response profile of gustducin-positive taste cells to multiple bitter compounds (quinine, denatonium, cyclohexamide, caffeine, sucrose octaacetate, tetraethylammonium, phenylthiourea, L-phenylalanine, $MgSO_4$, and high concentration of saccharin) was not significantly different between fungiform and circumvallate papillae. These bitter-sensitive taste cells were classified into several groups according to their responsiveness to multiple bitter compounds. Bitter responses of gustducin-positive taste cells were significantly suppressed by inhibitors of TRPM5 or PLCβ2. In contrast, several bitter inhibitors did not show any effect on bitter responses of taste cells. These results indicate that bitter-sensitive taste cells display heterogeneous responses and that TRPM5 and PLCβ2 are indispensable for eliciting bitter taste responses of gustducin-positive taste cells.

Key words: bitter receptor, breadth of responsiveness, taste coding, transgenic mouse, bitter antagonists

Abbreviations

BCML, Nα,Nα-bis(carboxymethyl)-L-Lysine; Caf, caffeine; Chx, cyclohexamide; CV, circumvallate papillae; Den, denatonium benzoate; FP, fungiform papillae; GABA, γ-aminobutylic acid; GFP, green fluorescent protein; GIV 3727, 4-(2,2,3-trimethylcyclopentyl) butanoic acid; IP_3R3, inositol-1,4,5-triophosphate receptor type 3; KO, knockout; L-Phe, L-phenylalanine; PLCβ2, phospholipase Cβ2; PTU, phenylthiourea; QHCl, quinine-HCl; Sac, saccharin-Na; SOA, sucrose octaacetate; Tas2rs, the type 2 taste receptors; TEA, tetraethylammonium; TPPO, triphenylphosphosphine oxide; TRPM5, transient receptor potential channel M5;

Introduction

Bitter taste protects animals from the ingestion of poisonous compounds. Many structurally diverse compounds such as alkaloids, terpenoids, flavonoids, phenylpropanes and thiols elicit bitter taste (Wiener et al., 2010). Bitter compounds are detected by the type 2 taste receptors (Tas2rs), which comprise a large G protein-coupled receptor family encoded by *Tas2r* genes (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000, Mueller et al., 2005). The number of functional *Tas2r* genes vary depending on the species with 25 in humans and 35 in mice (Go et al. 2005). Many of Tas2rs have had their cognate ligands identified in heterologous expression assays (Meyerhof et al. 2010; Lossow et al., 2016). These Tas2rs vary greatly in their breadth of tuning, ranging from very broadly to extremely narrowly tuned receptors.

In taste cells, binding of bitter compounds to Tas2rs activates the following signaling molecules: the heteromeric G-protein gustducin (Wong et al., 1996), phospholipase Cβ2 (PLCβ2, Zhang et al., 2003), inositol-1,4,5-triophosphate receptor type 3 (IP_3R3, Hisatsune et al., 2007) and transient receptor potential channel M5 (TRPM5, Zhang et al., 2003, 2007). Bitter-activated taste cells generate action potentials (Yoshida et al., 2006) and release neurotransmitters (Huang et al., 2007, Murata et al., 2010). Mice lacking these signaling molecules also showed diminished behavioral and neural responses to multiple bitter compounds (Wong et al., 1996; Zhang et al., 2003; Dotson et al., 2005; Damak et al., 2006; Hisatsune et al., 2007), suggesting that these signaling molecules are required for bitter taste responses. However, there is little evidence showing the contribution of these signaling molecules to bitter responses at the taste cell level *in situ*.

In taste buds, there are 4 types of taste cells (Type I ~ IV cells), which are characterized

by their morphology and expression pattern (Iwata et al., 2014). Bitter receptors and coupled transduction molecules are expressed in Type II cells (Yang et al., 2000; Perez et al., 2002; Clapp et al., 2004). Indeed, a subset of Type II cells in mouse fungiform (FP) and circumvallate (CV) papillae respond to bitter taste stimuli consistent with the expression pattern of receptors and transduction components for bitter taste (Tomchik et al., 2007; Yoshida et al., 2009a). However, several reports showed different response properties of bitter sensitive taste cells. Our previous study demonstrated that the majority of bitter sensitive cells in mouse FP responded to multiple bitter compounds (Yoshida et al., 2009a) whereas another study demonstrated that most bitter taste cells in rat CV respond to one or two of five bitter stimuli (Caicedo & Roper, 2001). Such discrepancy may be derived from methodological differences or from different locations of taste buds examined (FP vs CV) since gustatory nerve recordings showed different sensitivities to bitter compounds between the chorda tympani and the glossopharyngeal nerve (Ninomiya & Funakoshi, 1989; Ninomiya et al., 1991). The responsiveness of bitter sensitive taste cells has not been systematically compared between FP and CV using the same animal species and same experimental method.

Recent studies reported some bitter blockers for human Tas2rs. For example, 4-(2,2,3-trimethylcyclopentyl) butanoic acid (GIV 3727) inhibits six bitter taste receptors (Slack et al., 2010). Probenecid inhibits hTAS2R16, 38, and 43 (Greene et al., 2011). Sesquiterpene lactones and 6-methoxyflavanones block hTAS2R46 and hTAS2R39, respectively (Brockhoff et al., 2011; Roland et al., 2014). Some amino acid derivatives such as γ-aminobutylic acid (GABA) and Nα,Nα-bis(carboxymethyl)-L-Lysine (BCML) block hTAS2R4 (Pydi et al., 2014). These bitter blockers could be used to avoid unpleasant bitter taste of some medicines and health foods. These compounds were tested in heterologous expression system and some human psychophysics. But it is not clear whether these blockers inhibit activation

of bitter sensitive taste cells.

In this study, we focused on gustducin-positive mouse taste cells from both FP and CV and compared their responses to multiple bitter compounds. We also investigate the effect of pharmacological inhibitors for signaling molecules (PLCβ2 and TRPM5) in bitter sensitive taste cells and the effect of several bitter antagonists on activation of gustducin-positive taste cells.

Experimental Procedures

Animals

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the committee for Laboratory Animal Care and Use at Kyushu University, Japan. Subjects were adult (>8 weeks old) male and female transgenic mice expressing green fluorescent protein (GFP) under control of the gustducin promoter (gustducin-GFP mice, n=53) (Wong et al., 1999). All mice were housed under a 12:12-h light-dark cycle (lights on 0800-2000h) and had ad libitum access to tap water and food pellets (CE-2, CLEA Japan, Tokyo, Japan).

Taste cell recording

Recording procedures were similar to those used previously (Yoshida et al., 2006, 2009a, 2015) with some modifications to record responses from CV taste cells. Animals were sacrificed by cervical dislocation. The anterior (for FP preparation) and the posterior parts (for CV preparation) of the tongue were removed and injected with 50-100 μl of Tyrode solution containing 0.5-2 mg/ml elastase (Elastin Products, Owensville, MO). After incubation for 10-20 min at room temperature (25 ^{o}C), the lingual epithelium was

peeled and pinned out in a Sylgard coated culture dish. Individual FP or CV taste buds with a piece of surrounding epithelium were excised from this sheet and the mucosal side was drawn into the orifice of the stimulating pipette. The residual epithelial sheet was stored at 4 ^{o}C for another series of experiments. A gentle suction on the stimulating pipette was maintained to perfuse taste solutions and to hold the taste bud in place. Bath solution (Tyrode solution) was continuously flowed into the recording chamber with a peristaltic pump at approximately 2 ml/min. The receptor membrane was rinsed with distilled water at least 30 sec before and after taste stimulation (15 sec). Taste stimuli were applied to taste cells in randomized order. Taste bud cells containing GFP were identified by confocal laser scanning microscopy (FV-1000; Olympus, Tokyo, Japan) and were approached by a recording electrode (inner diameter ~1-3 μm, pipette resistances 1.5-3.5 MΩ). Seal resistances were typically 3-10 times the pipette resistances. Electrical signals were recorded by a high-impedance patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA) interfaced to a computer (Windows XP or 7) by an analog-to-digital board (Digidata 1320A; Axon Instruments). Signals were filtered at 1 KHz, sampled at 10 KHz and stored on the hard-disk drive of a computer using pCLAMP software (Gap-Free mode; Axon Instruments) for later analysis.

Solutions

Tyrode solution contained (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 $NaHCO_3$, 10 HEPES, 10 Glucose, 10 sodium pyruvate; pH adjusted to 7.4 with NaOH. Taste stimuli used were as follows (mM): 3 or 20 quinine-HCl (QHCl), 0.1 Chx, 10 denatonium benzoate (Den), 100 caffeine (Caf), 1 sucrose octaacetate (SOA), 100 tetraethylammonium (TEA), 100 $MgSO_4$, 100 L-phenylalanine (L-Phe), 1 phenylthiourea (PTU), 100 saccharin-Na (Sac), 1000 NaCl. Tastants were dissolved in

distilled water and used at room temperature (25 ^{o}C). Bitter antagonists GABA and BCML were added to 3 or 20 mM QHCl. The TRPM5 inhibitor triphenylphosphosphine oxide (TPPO), and PLC inhibitor U73122 and its inactive analog U73343, were prepared as 100, 5 and 5 mM stock solutions with dimethyl sulfoxide and dissolved in Tyrode solution, respectively. All chemicals were purchased from Sigma (St. Louis, MO) or Wako Pure Chemical Industries (Osaka, Japan).

Data analysis

To analyze data, the number of spikes per unit time was counted throughout the recording. The mean spontaneous impulse discharge for each unit was calculated by averaging the number of spikes over the 10 sec period that distilled water flowed over the taste pore prior to each stimulation. The final criteria for the occurrence of a response were the following: (1) Number of spikes was larger than the mean plus two standard deviations of the spontaneous discharge; (2) At least + 3 spikes were evoked by taste stimulation. The magnitude of response to a particular stimulus was obtained by counting the total number of impulses for the first 10 sec after the onset of stimulus application and subtracting the spontaneous impulse discharge.

Breadth of responsiveness of the taste cells was quantified using the following entropy equation (Smith and Travers, 1979; Travers and Smith, 1979).

$$H(\text{entropy}) = -K\sum_{i=1}^{n} p_i \log p_i$$

where H is the breadth of responsiveness, K is a scaling constant (1.0 for 10 bitter stimuli except for NaCl), p_i is the proportional response to each taste stimulus, and logarithms of pi are taken to the base ten. This entropy value varies continuously from 0.0 for a cell that responds exclusively to one stimulus to 1.0 for a cell that responds

equivalently to all of the taste stimuli.

One-way repeated ANOVAs with post hoc Bonferroni tests, paired t-test or Student's t-test were used to statistically evaluate difference of response magnitude between FP and CV, the effect of bitter antagonists, TRPM5 inhibitor and PLCβ2 inhibitor on bitter taste responses of taste cells. Correlation coefficients were calculated to analyze associations between responses to two bitter compounds. All statistical calculations and making of a dendrogram were performed using the statistical software packages IBM SPSS Statistics (IBM, Armonk, NY). All summarized data are presented as means ± 95% CI.

Results

Bitter responses of gustducin-positive taste cells

Alpha-gustducin (*Gnat3*)-knockout (KO) mice showed reduced behavioral and neural responses to bitter compounds as well as to sweet and umami tastants (Wong et al, 1996; He et al., 2004), suggesting that gustducin functions as a transduction component for bitter, sweet and umami taste. Indeed, gustducin-expressing mouse taste cells responded to bitter, sweet or umami compounds (Caicedo et al., 2003; Yoshida et al., 2009a). In order to analyze responsiveness of individual bitter-sensitive gustducin-positive taste cells to multiple bitter compounds, we used gustducin-GFP transgenic mice and recorded responses of gustducin-GFP+ taste cells to 10 bitter compounds (QHCl, Chx, Den, Caf, SOA, TEA, $MgSO_4$, L-Phe, PTU, high concentration of Sac), which were used in previous studies (Kuhn et al., 2004; Damak et al., 2006; Danilova and Hellekant, 2006; Yoshida et al., 2009a). We also tested high concentration of NaCl because responses to high concentrations of NaCl in bitter-sensitive Tas2r-expressing cells have been reported (Oka et al., 2013).

In both FP and CV, some gustducin-GFP+ taste cells showed specific responses to QHCl or Chx and some broadly responded to multiple bitter compounds (Fig. 1). It should be noted that a lower concentration (3 mM) of QHCl was used for CV taste cells because stable responses from CV taste cells were lost after stimulation by high concentrations (e.g. 20 mM) of QHCl. Bitter response profiles of each gustducin-GFP+ taste cell in FP and CV are shown in Figure 2. In these data, taste cells that only responded to Sac were excluded because these cells are sweet sensitive taste cells. The majority of bitter-sensitive gustducin-GFP+ taste cells responded to QHCl (27/30 cells in FP, 30/31 cells in CV), Chx (27/30 cells in FP, 25/31 cells in CV) and Den (18/30 cells in FP, 17/31 cells in CV). Other bitter compounds induced responses in a subset of bitter-sensitive gustducin-GFP+ taste cells ($MgSO_4$: 7/30 cells in FP, 2/31 cells in CV; TEA: 10/30 cells in FP, 5/30 cells in CV; Caf: 6/30 cells in FP, 11/31 cells in CV; L-Phe: 4/30 cells in FP, 4/31 cells in CV; PTU: 4/30 cells in FP, 2/31 cells in CV; Sac: 3/30 cells in FP, 6/31 cells in CV; SOA: 2/30 cells in FP, 2/31 cells in CV). Some bitter-sensitive gustducin-GFP+ taste cells also responded to high concentrations of NaCl (6/26 cells in FP, 2/31 cells in CV).

Comparison of bitter responses of gustducin-GFP+ taste cells from FP vs. CV

We compared bitter responses of gustducin-GFP+ taste cells from FP and CV. In both FP and CV, gustducin-GFP+ taste cells responded to 1-9 bitter compounds from among the 10 compounds tested. The mean entropy value for FP and CV taste cells, which represents the breadth of responsiveness, was 0.462 ± 0.072 and 0.452 ± 0.072, respectively ($P>0.1$, Student's t-test). Thus the breadth of responsiveness of bitter-sensitive gustducin-GFP+ taste cells did not differ between FP and CV.

To determine if there are specific groups of bitter-sensitive gustducin-GFP+ taste cells in FP or CV, we classified the cells by hierarchical cluster analysis according to their

response profiles. As shown in Figure 3, bitter-sensitive gustducin-GFP+ taste cells were largely divided into 5 groups. The Chx group had large responses to Chx and a relatively small mean entropy value (0.275 ± 0.091, n=11). Many cells in this group (8/11 cells) also responded to QHCl. The QHCl group had large responses to QHCl and a relatively small mean entropy value (0.263 ± 0.100, n=11). The CQC group was characterized by large responses to Chx, QHCl and Caf (except for FP8) and a relatively large mean entropy value (0.600 ± 0.112, n=8). The DQ group showed large responses to Den and QHCl (except for FP28). The mean entropy value for the DQ group (0.456 ± 0.137, n=7) is almost the same as that for all FP or CV bitter sensitive cells. The DQC group was characterized by large responses to Den, QHCl and Chx (except for CV8) and a relatively large mean entropy value (0.582 ± 0.035, n=24). About 40% of bitter-sensitive gustducin-GFP+ taste cells (24/61 cells) were classified into this group. All of these groups contained both FP and CV taste cells, suggesting that there is no specific group of bitter sensitive cells only in FP or only in CV. Response properties of bitter-sensitive gustducin-GFP+ cells may not differ between FP and CV.

We also compared the mean magnitude of response to some bitter compounds in gustducin-GFP+ taste cells between FP and CV (Fig. 4). We excluded responses to QHCl from this analysis because we used different concentration (3 mM in CV vs 20 mM in FP). Responses to $MgSO_4$ and NaCl were significantly larger in FP than in CV taste cells ($P<0.05$, Student's t-test). Responses to other bitter compounds were not significantly different between FP and CV taste cells ($P>0.05$, Student's t-test).

Association between responses to two bitter compounds in gustducin-GFP+ taste cells

Next we determined if responses to any two bitter compounds were associated in particular gustducin-GFP+ taste cells. We calculated correlation coefficients between responses to two bitter compounds in FP and CV taste cells (Table 1). In both FP and

CV, there is a strong positive correlation between responses to QHCl and Chx, both of which elicited responses in many gustducin-GFP+ taste cells. Responses to $MgSO_4$ and high concentration of NaCl were also well associated in both FP and CV taste cells, although the numbers of cells responding to these compounds were relatively small ($MgSO_4$: 7/30 cells in FP, 2/31 cells in CV; 6/26 cells in FP, 2/31 cells in CV). Weak but significant correlations were observed in responses to PTU and Sac, $MgSO_4$ and Sac, $MgSO_4$ and L-Phe in both FP and CV taste cells. These associations between responses to two bitter compounds suggest similarities of receptors and/or cells responsible for responses to these pairs of bitter compounds.

Effect of several bitter antagonists on bitter responses of gustducin-GFP+ taste cells

Recent studies reported that some amino acid derivatives function as bitter antagonists (Pydi et al., 2014). In HEK cells expressing human TAS2R4, GABA and BCML effectively suppressed responses to QHCl (IC_{50} = 3.2 ± 0.3 μM and 59 ± 18 nM respectively). Because many gustducin-GFP+ taste cells responded to QHCl, we tested whether GABA and BCML suppressed responses to QHCl in gustducin-GFP+ taste cells. We used 100 μM GABA and 10 μM BCML, both of which almost completely suppressed QHCl responses in HEK cells expressing human TAS2R4 (Pydi et al., 2014). GABA and BCML were mixed with QHCl solutions and responses to QHCl with and without antagonists were compared (Fig. 5). Responses of gustducin-GFP+ taste cells to QHCl were not affected by the addition of GABA (Fig. 5A, B, $P>0.1$, n=10, paired t-test). BCML also had no effect on QHCl responses of gustducin-GFP+ taste cells (Fig. 5C, D, $P>0.1$, n=10, paired t-test). Thus we did not observe any effect of GABA and BCML on QHCl responses of gustducin-GFP+ taste cells in mice.

Effect of pharmacological blockers of TRPM5 and PLCβ2

PLCβ2-KO mice and TRPM5-KO mice showed reduced behavioral and neural responses to bitter compounds as well as to sweet and umami taste stimuli (Zhang et al, 2003; Dotson et al., 2005; Damak et al., 2006). Thus, both PLCβ2 and TRPM5 play critical roles in bitter taste responses. Although contributions of PLCβ2 and TRPM5 to bitter responses is apparent from these behavioral experiments and gustatory nerve recordings, there is little evidence directly demonstrating the role of PLCβ2 and TRPM5 in bitter-sensitive taste cells. Using pharmacological blockers for PLCβ2 and TRPM5, we examined contribution of these transduction molecules to bitter taste responses in gustducin-GFP+ taste cells. Bitter taste responses of gustducin-GFP+ taste cells were almost completely and irreversibly eliminated 5-10 min after bath application of the PLC blocker 5 μM U73122 ($F_{(2,6)}$=18.788, P<0.01, One-way repeated ANOVA; P<0.01 post hoc Bonferroni test; Fig. 6A, B). In contrast, application of the inactive analog U73343 did not affect bitter responses of Gustducin-GFP+ taste cells (n=5, P>0.1, paired t-test, Fig 6C, D). The TRPM5 blocker TPPO (30 μM) almost completely inhibited bitter taste responses of gustducin-GFP+ taste cells (Fig. 6E, F). The effect of TPPO was reversible ($F_{(2,6)}$=22.445, P<0.001, One-way repeated ANOVA; P<0.01, post hoc Bonferroni test), and concentration dependent (Fig. 6G, n=7-10, P<0.05-0.001, vs control, paired t-test). These results suggest that both PLCβ2 and TRPM5 are indispensable for eliciting bitter taste responses in gustducin-GFP+ taste cells.

Discussion

We used the same experimental method for recording bitter taste responses from both FP and CV gustducin-GFP+ taste cells, and found that response profiles of bitter-sensitive gustducin-GFP+ taste cells were not significantly different between FP and CV (Fig. 2, 3). The breadth of responsiveness of bitter-sensitive gustducin-GFP+

taste cells in FP was similar to that in CV. There was no specific group of bitter-sensitive gustducin-GFP+ taste cells in FP vs. CV (Fig. 3). Thus, the overall response profiles of gustducin-GFP+ taste cells to multiple bitter compounds are likely quite similar between FP and CV.

Previous calcium imaging experiments demonstrated that most bitter taste cells in rat CV respond to one or two of five bitter stimuli (QHCl, Chx, Den, SOA and PTC; Caicedo & Roper, 2001). Twenty-three of 69 (33%) rat CV bitter sensitive cells showed multiple sensitivities to bitter compounds, whereas 29 of 31 (94%) mouse CV bitter sensitive cells in this study showed responses to multiple bitter compounds. These two studies used different experimental methods (calcium imaging vs electrophysiological recording), different animals (rat vs mouse), different numbers of bitter compounds (5 vs 10 compounds) and different taste cells (randomly recorded cells vs gustducin-positive cells identified by GFP expression), all of which may contribute to different results between two studies. It is important to note that our data do not include bitter-sensitive taste cells which do not express gustducin. A previous study demonstrated that approximately half of the bitter-sensitive taste cells in mouse CV taste buds express gustducin (Caicedo et al., 2003), suggesting the existence of gustducin-negative bitter-sensitive taste cells. Responses of gustducin-negative taste cells have not been investigated in this study, but it is possible that these cells may have different response profiles from those of gustducin-positive taste cells. In any case, both studies demonstrated that Chx, QHCl and Den were the stimuli exciting most of bitter sensitive taste cells.

Bitter-sensitive gustducin-GFP+ taste cells were classified into groups according to their response profiles (Fig. 3). Each of these groups could contribute to discrimination among bitter compounds. For example, the Chx and QHCl groups showed relatively specific responses to Chx and QHCl, therefore, may play a role in identifying Chx and

QHCl, respectively. In other basic tastes, salt sensitive taste cells and fibers can be classified into two groups according to their sensitivities to the epithelial sodium channel (ENaC) blocker amiloride (Ninomiya, 1996; Yoshida et al., 2009b). Umami sensitive cells and fibers can be classified into 4 groups according to their sweet responses and synergism with umami compounds (Niki et al., 2011; Yasumatsu et al., 2012). Such specific coding channels for taste information from taste receptor cells to the central nervous system would underlie discrimination among these tastes. Similarly to these cases, bitter-sensitive gustatory fibers could be classified into several groups corresponding to gustducin-GFP+ taste cells and could consist of bitter coding channels. Future studies would be useful to test this possibility.

Patterns of expression of bitter taste receptors genes would shape the response profiles of each bitter sensitive taste cell. One study demonstrated that individual taste cells in rats express multiple Tas2rs (Adler et al. 2000), suggesting that bitter sensitive taste cells can detect multiple bitter compounds. Other studies using mice and human samples demonstrated that individual taste cells show a more limited coexpression of Tas2rs (Matsunami et al. 2000, Behrens et al. 2007), suggesting heterogeneous populations of bitter taste cells. In this study, we demonstrated that response patterns of bitter-sensitive gustducin-positive taste cells are heterogeneous, supporting the idea of heterogeneous populations of bitter taste cells. Bitter sensitive taste cells in Chx and QHCl groups showed relatively small mean entropy values, indicating that they may possess more limited sets of Tas2rs. In contrast, bitter sensitive taste cells in the DQC and CQC groups showed broad responsiveness to multiple bitter compounds, therefore, these cells may express a large set of Tas2rs. The majority of bitter sensitive taste cells responded to Chx and QHCl. In a study examining heterologously expressed taste receptors, QHCl activated seven mouse Tas2rs (Tas2r105, 108, 115, 126, 137, 140 and 144), whereas Chx activated only Tas2r105 (Lossow et al., 2016). Thus, Chx receptor

Tas2r105 and/or other receptors for QHCl such as Tas2r108, 115, 126, 137, 140 and 144 could be expressed broadly among bitter-sensitive taste cells. Caf and SOA elicited responses in a smaller subset of bitter sensitive taste cells, suggesting limited expression of Caf receptor (Tas2r121) and SOA receptor (Tas2r117) among bitter-sensitive taste cells. Responses to $MgSO_4$ were strongly correlated with responses to high concentrations of NaCl both in FP and CV (Table 1). These ionic compounds may activate the same bitter taste receptor(s), although it has not been identified. In future studies, the expression pattern and level of Tas2rs in individual taste cells may be defined by single cell RNA-seq.

Previous studies reported some bitter inhibitors for human TAS2Rs: GIV3727 inhibited activation of six bitter receptors (hTAS2R4, 7, 31, 40, 43, and 49) (Slack et al., 2010). Probenecid inhibited hTAS2R16, 38, and 43 (Greene et al., 2011). Sesquiterpene lactones and 6-methoxyflavanones blocked hTAS2R46 and hTAS2R39, respectively (Blockhoff et al., 2011; Roland et al., 2014). GABA and BCML inhibited hTAS2R4 (Pydi et al., 2014). Among these bitter inhibitors, we chose to use GABA and BCML because these amino acid derivatives have been reported to inhibit QHCl responses, which were easily recorded from gustducin-GFP+ taste cells. However, both GABA and BCML did not show any effect on QHCl responses of gustducin-GFP+ taste cells (Fig. 5). Species differences may be one possible explanation for this discrepancy: for example, gymnemic acid inhibits human but not mouse sweet taste receptor T1R2/T1R3 (Sanematsu et al., 2014) while PTC is a well-known agonist for human TAS2R38 but does not activate the mouse ortholog Tas2r138 at <0.1 mM (Lossow et al., 2016). Thus, activation/inhibition properties may not correspond well with human and mouse TAS2R orthologs. The mouse ortholog for hTAS2R4 (Tas2r108) could be activated by QHCl (Lossow et al., 2016), therefore GABA and BCML may not inhibit activation of this receptor. In heterologous system, QHCl activates multiple Tas2rs (Lossow et al., 2016).

This raises another possibility that the contribution of Tas2r inhibition by GABA and BCML (maybe Tas2r108) is too small to affect QHCl responses in gustducin-GFP+ taste cells. Instead, activation of other receptors (such as Tas2r105, 115, 126, 137, 140 and 144) by QHCl may account for the most part of QHCl responses in gustducin-GFP+ taste cells.

Gustducin-KO, PLCβ2-KO and TRPM5-KO mice show diminished behavioral and neural responses to bitter (as well as sweet and umami) stimuli (Wong et al., 1996; Zhang et al, 2003; He et al., 2004; Dotson et al., 2005; Damak et al., 2006), indicating that bitter signaling pathway utilizes α-gustducin, PLCβ2 and TRPM5. Our results using pharmacological blockers for PLCβ2 and TRPM5 were consistent with these previous studies (Fig. 6). Thus, gustducin-PLCβ2-TRPM5 pathway is essential for activation of gustducin-positive taste cells by bitter compounds. Some KO studies demonstrated residual responses to bitter compounds (Wong et al., 1996; Dotson et al., 2005; Damak et al., 2006), suggesting the existence of bitter signaling components or pathways independent of gustducin, PLCβ2 and TRPM5. Caicedo et al (2003) showed gustducin-independent bitter responses in mouse CV taste cells and possible involvement of Gαi2 in bitter taste transduction. Taken together, there may be two types of bitter sensitive taste cells; gustducin-positive cells that use a gustducin-PLCβ2-TRPM5 pathway and gustducin-negative cells that use other transduction components such as Gαi2. Further studies are required for the characterization of gustducin-negative bitter-sensitive taste cells in mice.

In conclusion, the present study investigated bitter taste responses of gustducin-positive taste cells in mouse FP and CV. Overall response profiles of gustducin-GFP+ taste cells to multiple bitter compounds were not significantly different between FP and CV. These bitter sensitive taste cells were classified into several groups, suggesting that bitter taste cells are heterogeneous from the point of view of their response properties. Such

heterogeneity may contribute to discrimination among bitter compounds.

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Conflicts of interest

The authors declare no competing financial interests.

Author contributions

RY conceived and designed the experiments. RY, ST, and KS performed the experiments. RY, ST, KS, NS and YN analyzed the data. RFM contributed reagents/materials/analysis tools. RY, RFM and YN wrote the paper.

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Figure 1. Sample recordings from gustducin-GFP+ taste cells to bitter stimuli. Top images show gustducin-GFP+ taste cells from which taste responses were recorded. Lower panels show sample recordings of taste responses to 1000 mM NaCl, 1 mM SOA, 100 mM Sac, 1 mM PTU, 100 mM L-Phe, 100 mM Caf, 100 mM TEA, 100 mM $MgSO_4$, 10 mM Den, 20 (FP) or 3 (CV) mM QHCl, 0.1 mM Chx. Taste stimuli presentations were indicated by horizontal black bars. FP: fungiform taste cell. CV circumvallate taste cell.

Figure 2. Response profiles of 30 (FP, left) and 31 (CV, right) bitter-sensitive gustducin-GFP+ taste cells. Taste responses are shown for each taste cell to 0.1 mM

Chx, 20 mM (FP) or 3 mM (CV) QHCl, 20 mM Den, 100 mM $MgSO_4$, 100 mM TEA, 100 mM Caf, 100 mM L-Phe, 1 mM PTU, 100 mM Sac, 1 mM SOA, 1000 mM NaCl. Taste cells are arranged according to the number of bitter taste stimuli to which they responded.

Figure 3. Cluster analysis of bitter-sensitive gustducin-GFP+ taste cells. Right panel shows a dendrogram produced by hierarchical clustering based on response profiles of bitter-sensitive taste cells. Left panel shows response profiles of each bitter-sensitive taste cell. Cell numbers correspond to those in Figure 2. Darker, larger ovals correspond to greater percentages of the maximal response. The Chx group showed large responses to Chx. The QHCl group showed large responses to QHCl. The CQC group showed large responses to QHCl, Caf and Chx. The DQ group showed large responses to Den and QHCl. The DQC group showed large responses to Den, QHCl and Chx.

Figure 4. Mean response amplitudes of gustducin-GFP+ taste cells to various bitter compounds. Responses of FP (grey bars) and CV (white bars) gustducin-GFP+ taste cells to various bitter compounds are shown. Responses to NaCl and $MgSO_4$ were significantly smaller in CV than in FP taste cells. Responses to QHCl were greater in CV than in FP taste cells (n=30-31, *: $P<0.05$, Student's t-test). Data shown are means ± 95% CI.

Figure 5. The effects of bitter antagonists on responses of gustducin-GFP+ taste cells. A, B. Sample recordings (A) and summarized data (B) showing the effect of GABA on QHCl responses of gustducin-GFP+ taste cells. GABA did not affect QHCl responses of taste cells (n=10, $P>0.1$, paired t-test). C, D. Sample recordings (C) and summarized data (D) showing the effect of Nα,Nα-bis(carboxymethyl)-L-Lysine (BCML) on QHCl

responses of gustducin-GFP+ taste cells. BCML also did not affect QHCl responses of taste cells (n=10, P>0.1, paired t-test). Data shown are means ± 95% CI.

Figure 6. The effect of inhibitors of PLCβ2 and TRPM5 on bitter responses of gustducin-GFP+ taste cells. A, B. Sample recordings (A) and summarized data (B) showing the effect of bath application of 5 μM U73122 on bitter taste responses of gustducin-GFP+ taste cells. U73122 irreversibly inhibited bitter taste responses of taste cells ($F_{(2,6)}$=18.778, P<0.01, One-way repeated ANOVA; **: P<0.01, post hoc Bonferroni test). C, D. Sample recordings (C) and summarized data (D) showing the effect of bath application of 5 μM U73343 on bitter taste responses of gustducin-GFP+ taste cells. U73343 had no effect on bitter taste responses of taste cells (n=5, P>0.1, paired t-test). E, F. Sample recordings (E) and summarized data (F) showing the effect of bath application of 30 μM TPPO on bitter taste responses of gustducin-GFP+ taste cells. TPPO reversibly inhibited bitter taste responses of taste cells ($F_{(2,6)}$=22.445, P<0.001, One-way repeated ANOVA; **P<0.01, ***P<0.001, post hoc Bonferroni test). G. Concentration dependent effect of TPPO on bitter responses of taste cells. Asterisks indicate significant difference from control (n=7-10, *P<0.05, ***P<0.001, paired t-test). Data shown are means ± 95% CI.

Table 1. Association between responses to two bitter compounds in individual taste cells

		FP										
		NaCl	SOA	Sac	PTU	L-Phe	Caf	TEA	$MgSO_4$	Den	QHCl	Chx
CV	NaCl		**.442** **.024** **26**	.062 .762 26	.146 .478 26	.224 .272 26	-.053 .798 26	.109 .596 26	**.870** **.000** **26**	.213 .297 26	.091 .657 26	.002 .991 26
	SOA	-.063 .735 31		**.643** **.000** **30**	**.739** **.000** **30**	.200 .288 30	-.113 .552 30	.235 .212 30	**.728** **.000** **30**	.349 .059 30	**.504** **.005** **30**	.321 .084 30
	Sac	**.575** **.001** **31**	-.110 .556 31		**.438** **.015** **30**	.125 .510 30	.036 .850 30	.127 .503 30	**.459** **.011** **30**	.278 .137 30	**.411** **.024** **30**	.242 .197 30
	PTU	.324 .075 31	-.063 .735 31	**.434** **.015** **31**		.084 .658 30	.114 .550 30	.169 .371 30	**.477** **.008** **30**	.247 .188 30	**.558** **.001** **30**	.301 .106 30
	L-Phe	**.472** **.007** **31**	**.434** **.015** **31**	**.511** **.003** **31**	.150 .419 31		-.094 .623 30	.037 .846 30	**.376** **.040** **30**	.015 .938 30	.201 .287 30	.274 .143 30
	Caf	.155 .406 31	-.085 .649 31	.319 .080 31	-.016 .933 31	.084 .654 31		.047 .805 30	-.108 .569 30	.041 .832 30	-.005 .979 30	-.112 .557 30
	TEA	.346 .057 31	.160 .391 31	**.515** **.003** **31**	.091 .625 31	.327 .073 31	.118 .528 31		.258 .169 30	**.398** **.029** **30**	**.621** **.000** **30**	**.491** **.006** **30**
	$MgSO_4$	**.970** **.000** **31**	-.068 .717 31	**.482** **.006** **31**	.272 .139 31	**.395** **.028** **31**	.107 .566 31	.283 .123 31		**.486** **.007** **30**	**.558** **.001** **30**	**.513** **.004** **30**
	Den	-.118 .527 31	.166 .372 31	.017 .930 31	.037 .843 31	.234 .206 31	-.315 .084 31	-.194 .296 31	-.141 .449 31		**.441** **.015** **30**	**.534** **.002** **30**
	QHCl	-.020 .915 31	.049 .795 31	-.040 .833 31	-.197 .288 31	.024 .897 31	.068 .715 31	-.053 .777 31	-.003 .986 31	.223 .227 31		**.621** **.000** **30**
	Chx	-.095 .612 31	-.026 .890 31	.033 .859 31	-.201 .278 31	-.051 .787 31	.276 .132 31	.079 .673 31	-.138 .459 31	.268 .145 31	**.731** **.000** **31**	

Upper: correlation coefficient, middle: p value, lower: n

Bold + underline shows significant correlation ($P<0.05$).

Fig.1 Yoshida et al

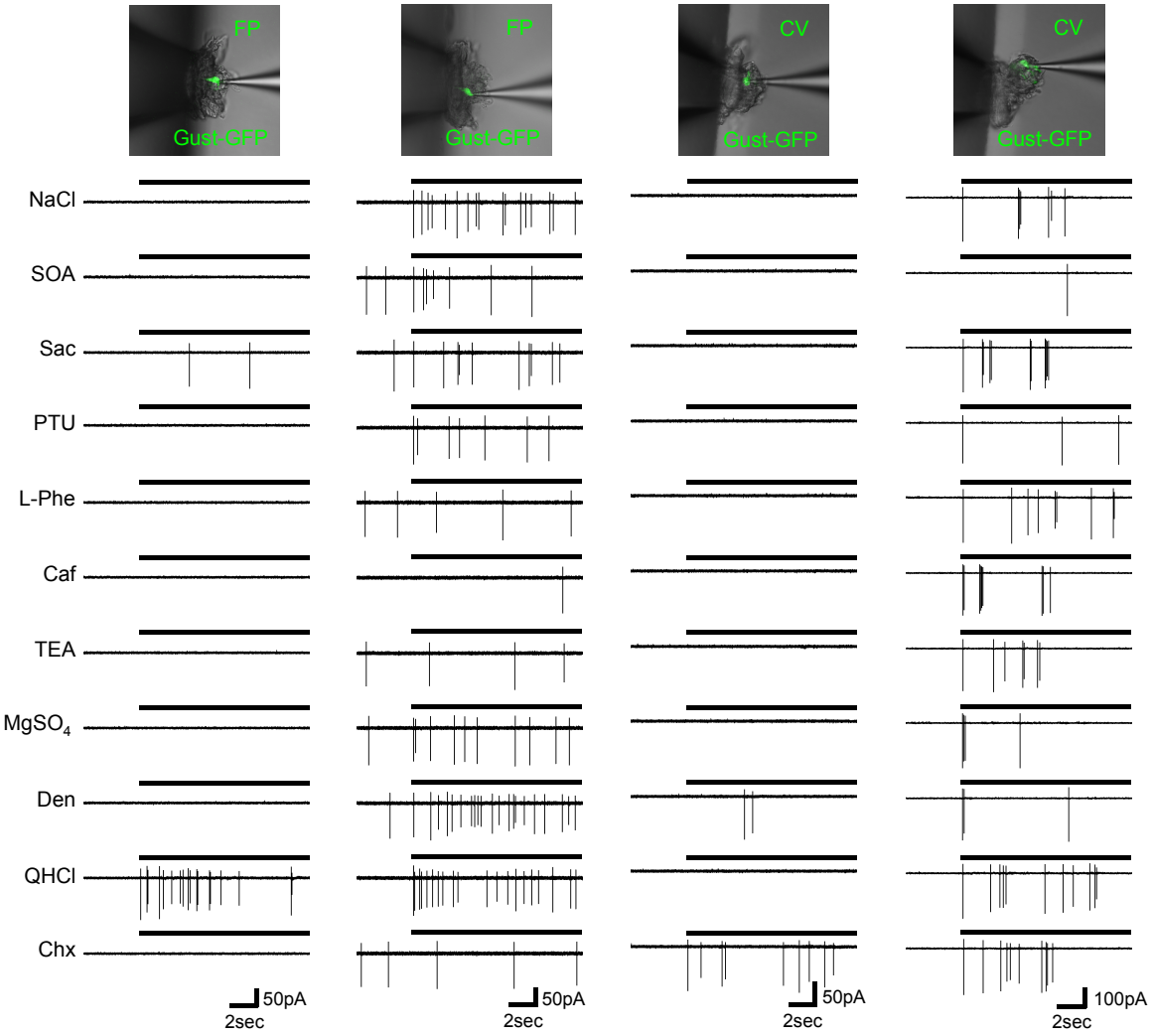

Fig.2 Yoshida et al

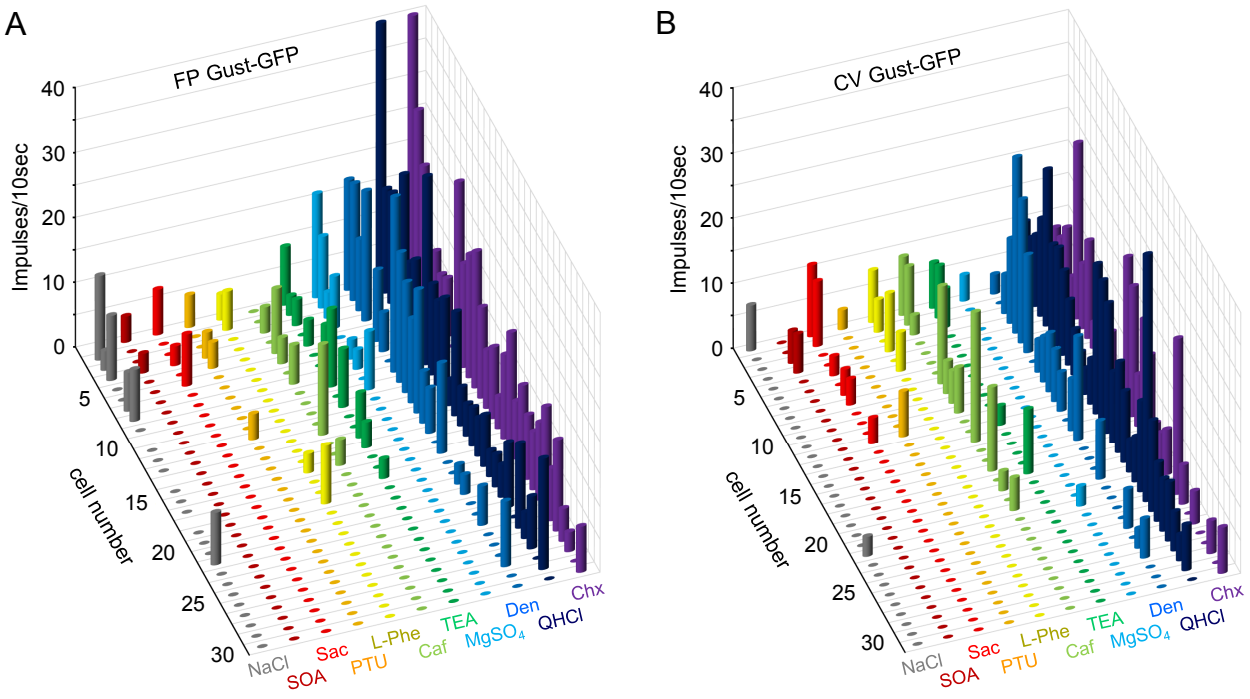

Fig.3 Yoshida et al

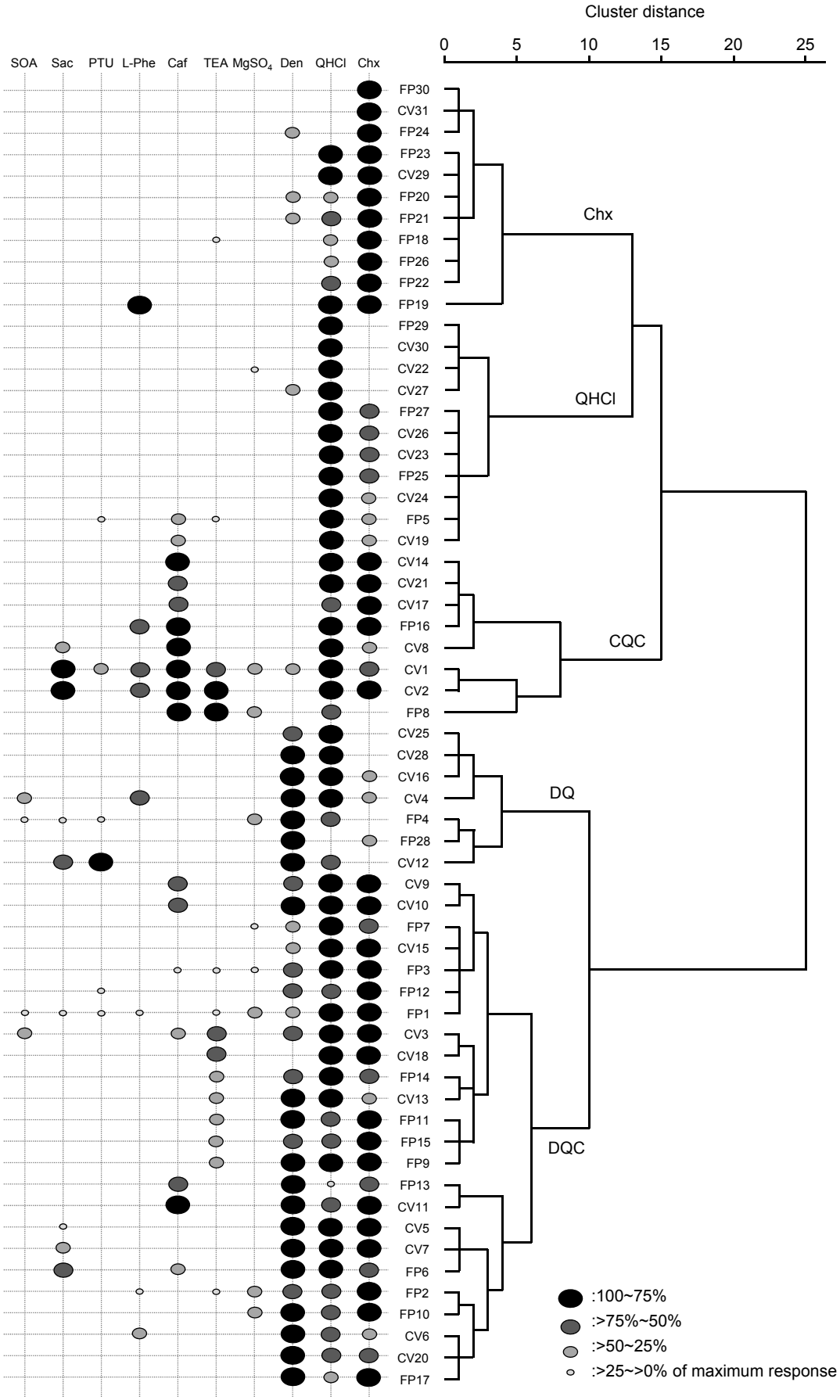

Fig.4 Yoshida et al

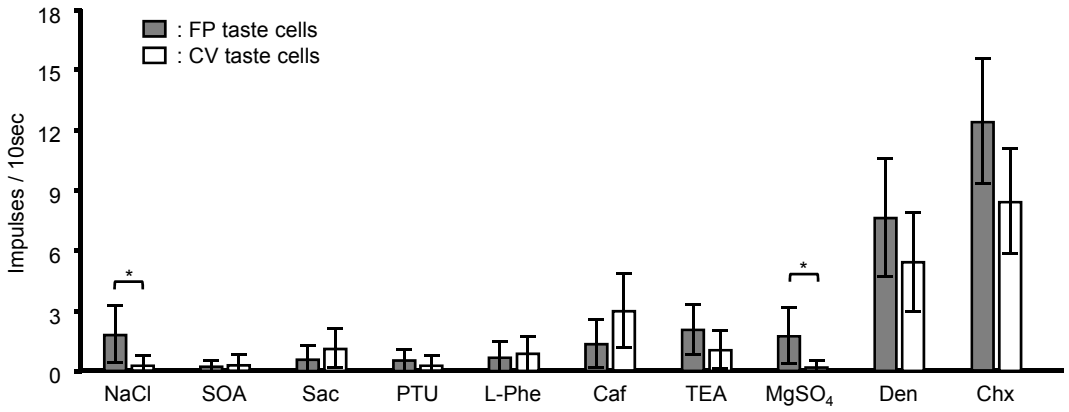

Fig.5 Yoshida et al

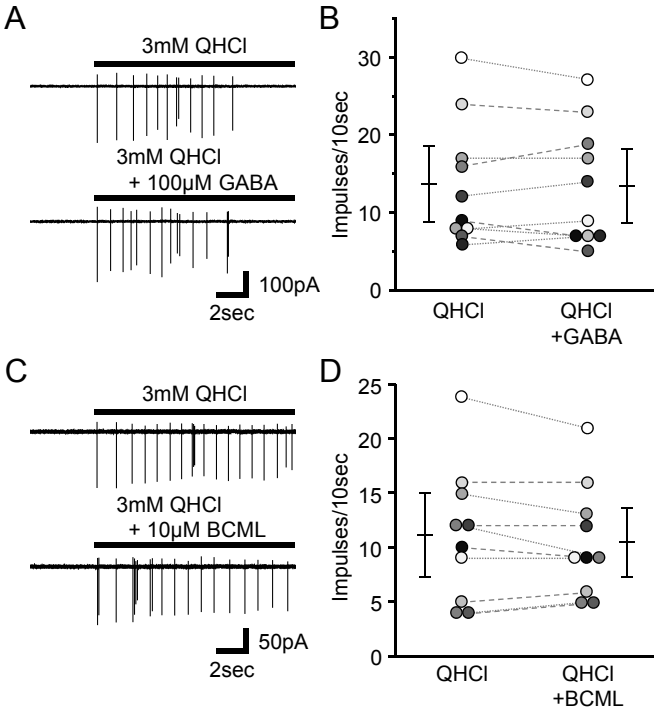

Fig.6 Yoshida et al